Efficacy of Organic Acids in Hand Cleansers for Prevention of Rhinovirus Infections

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Direct hand-to-hand contact is an important mechanism of transmission of rhinovirus infection. The rhinoviruses are inactivated at a low pH. A survey of organic acids in vitro revealed that these compounds have antirhinoviral activity that persists for at least 3 h after application to the skin. In additional studies of salicylic acid (SA) and pyrogulutamic acid (PGA), the hands of volunteers were contaminated with rhinovirus at defined times after application of the acid, and then volunteers attempted to inoculate the nasal mucosa with one hand and quantitative viral cultures were done on the other hand. In one study, 3.5% SA or 1% SA with 3.5% PGA was compared with controls 15 min after application to assess the efficacy of the inactivation of virus and prevention of infection. Virus was recovered from the hands of 28 out of 31 (90%) of the volunteers in the control group compared to 4 out of 27 (15%) and 0 out of 27 in the groups administered 3.5% and 1% SA, respectively (P < 0.05). Rhinovirus infection occurred in 10 out of 31 (32%) of the controls and 2 out of 27 (7%) in the groups administered 3.5% and 1% SA, respectively (P < 0.05). To show the efficacy of acids in reducing the person-to-person transmission of rhinovirus infection under experimental conditions (2, 10), but the efficacy of the viruses in a natural setting was never studied. The purpose of these studies was to assess the residual virucidal activity of various organic acids used in currently available over-the-counter skin products and to determine the efficacy of acids with virucidal activity for the prevention of experimental rhinovirus colds.

Rhinoviruses, the causative agent in at least one-half of all common colds, are spread primarily by direct person-to-person contact (6, 11). The virus survives for up to 3 h on skin and can be recovered from the hands of approximately 40% of infected individuals (13, 17). Simple hand washing or the use of a variety of virucidal agents will remove infectious virus from the hands (12), but the practical use of a virucidal agent for prevention of colds will require an agent that has virucidal activity that persists for an extended period of time after application. The only virucidal agents that have been reported to have residual activity are glutaric acid, which has virucidal activity that persists for up to 6 h (8), and 2% aqueous iodine, which has virucidal activity that persists for at least 2 h after application (7, 12). When used as a hand treatment, iodine has been shown to interrupt transmission of experimental rhinovirus colds and reduce the incidence of colds in the natural setting (7, 11). Although these studies with iodine demonstrate the feasibility of the interruption of rhinovirus transmission for the prevention of colds, iodine is not an acceptable virucidal agent for general use.

The inactivation of rhinovirus by acids is well known and has been used for many years to distinguish the rhinoviruses from other picornaviruses. The mechanism of this inactivation appears to involve changes in the virion structure similar to those that occur during virus uncoating. This conformational change results in a loss of VP4 with an associated loss of infectivity (3). This acid sensitivity has been used as the basis for attempts to develop effective virucidal agents against the rhinovirus. A combination of citric acid, malic acid, and sodium lauryl sulfate incorporated into a nasal tissue is virucidal for a broad spectrum of rhinovirus serotypes (2, 9). Use of these tissues reduced the person-to-person transmission of rhinovirus infection under experimental conditions (2, 10), but the efficacy of the viruses in a natural setting was never studied. The purpose of these studies was to assess the residual virucidal activity of various organic acids used in currently available over-the-counter skin products and to determine the efficacy of acids with virucidal activity for the prevention of experimental rhinovirus colds.

MATERIALS AND METHODS

Virus. Rhinovirus type 39 was used for these studies. Stock pools of rhinovirus type 39 were produced in HeLa cells (American Type Culture Collection, Manassas, Va.). The virus pools were aliquoted and stored frozen at −80°C until use. Virus titers in specimens recovered from collagen substrates and treated fingers were calculated as the 50% tissue culture infective dose (TCID50).

Assessment of residual antiviral activity on collagen substrates. An in vitro assay (modified from a previously described method [18]) was used to measure the antiviral activity of various compounds. Twenty microliters of each compound was applied to 1 cm² of a skin collagen substrate (Vitro-Skin; IMS Inc., Milford, Conn.) and allowed to dry. The substrate contains both protein and lipid components and is designed to have topography, pH, critical surface tension, and ionic strength similar to those of human skin. After 10 min, 1 h, or 3 h, the treated areas were inoculated with 10⁴ TCID50 of rhinovirus. After a 10-min contact time, the virus was eluted from the substrate and quantitated. For experiments comparing organic acids, the acids were standardized to 0.145 M in a 1:1 mixture of water and ethanol, and the pH of each solution was adjusted to 3.0 by using hydrochloric acid. A water-ethanol vehicle

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(adjusted to pH 3 with hydrochloric acid) was used as the negative control, and 2% iodine was used as a positive control.

Assessment of residual antiviral activity on human skin. The antiviral activities of various hand cleansers were assessed by a published standardized method (18), with minor modifications. Subjects initially washed their hands and wrists with soap and water, rinsed and dried them. The hands and/or inner forearms were then treated with 70% ethanol and air dried. Finger cots were placed over the thumbs to prevent treatment of this area, and then 1.7 mL of the test material was applied to the hands and forearms and allowed to dry. The residual virucidal activity was determined 10 min, 1 h, and 3 h after application by applying 10 μL of a suspension containing approximately 10^9 TCID<sub>50</sub> of rhinovirus to treated or untreated skin. After 7 to 10 min, the virus was eluted from the skin by repeatedly inverting a plastic vial containing 1 mL of tissue culture medium with the mouth of the vial pressed against the skin site of interest. Studies on eluate from treated skin sites that were not inoculated with virus confirmed that the eluate was not virucidal. Subjects in these experiments were allowed to perform normal activities but were instructed not to wash their hands or wear gloves during the experiment.

Data analysis. For each product tested, duplicate values at each time point were log transformed and averaged. Results are expressed as the average log reduction in TCID<sub>50</sub> per milliliter compared to untreated controls.

Experimental infection studies with human volunteers. (i) Subjects. The experimental infection studies were conducted by Hill Top Research, Inc., Winnipeg, Manitoba, Canada. Subjects were recruited from Winnipeg and surrounding communities for participation. Subjects were required to be in good health and from 18 to 60 years old. In addition, subjects were required to have a serum-neutralizing antibody titer of more than 1:4 to the study virus. Subjects with abnormal nasal anatomy or mucosa, a respiratory tract infection, or symptomatic allergic rhinitis in the previous 2 weeks; pregnant or lactating females or females not on medically approved birth control; and subjects allergic to personal cleaning products or salicylates were excluded. Subjects were compensated for participation. This study was reviewed and approved by the Ethics Review Board of the ethical review committee. All volunteers gave written informed consent for participation.

(ii) Challenge virus. The challenge virus used for this study was rhinovirus type 39. This challenge pool has been safety tested according to consensus guidelines (5). The target inoculum of challenge virus was 100 TCID<sub>50</sub> contained in a volume of 100 μL. This inoculum was selected based on previously unpublished data that suggested that this method of challenge would produce an infection rate of approximately 50% in the placebo treatment group.

(iii) Virologic assays. Virus shedding was detected by virus isolation in cell culture. Nasal wash specimens were collected by instillation of 5 mL of 0.9% saline into each nostril. This wash was then expelled into a waxed paper cup, aliquoted into vials, and stored frozen at −80°C until it was processed for viral culture. Each specimen was inoculated into two tubes of human embryonic lung fibroblast cells and incubated on roller drums at 33°C for 14 days. Rhinovirus was identified by the development of typical cytopathic effects. Subjects with a positive viral culture on any of the postchallenge study days were considered infected. Virus titers in the original hand rinse specimens were determined from viral culture. Nasal wash specimens were collected by instillation of 5 mL of 0.9% saline into each nostril. This wash was then expelled into a waxed paper cup, aliquoted into vials, and stored frozen at −80°C until it was processed for viral culture. Nasal wash specimens were collected by instillation of 5 mL of 0.9% saline into each nostril. This wash was then expelled into a waxed paper cup, aliquoted into vials, and stored frozen at −80°C until it was processed for viral culture. Nasal wash specimens were collected by instillation of 5 mL of 0.9% saline into each nostril. This wash was then expelled into a waxed paper cup, aliquoted into vials, and stored frozen at −80°C until it was processed for viral culture.

(iv) Conduct of clinical trial 1. Clinical trial 1 was conducted by using a double-blind, randomized design. Qualified subjects were randomized to treatment with vehicle (62% ethanol, 1% ammonium lauryl sulfate, and 1% KIucel), vehicle containing 3.5% salicylic acid, or vehicle containing 1% salicylic acid and 3.5% pyrogallic acid. The volunteers' hands were disinfected, and then the test product (1.7 mL) was applied to both hands of each subject and allowed to dry. Fifteen minutes after application of the test material, the virus was completely dry, the fingertips of each hand were contaminated with 155 TCID<sub>50</sub> of rhinovirus type 39 in a volume of 100 μL. After contamination with virus, the hands were allowed to air dry for 10 min before the volunteers attempted to intentionally inoculate the virus by making contact between the fingers and both the conjunctiva and the nasal mucosa. This inoculation was done only with the right hand, and a consistent method was used to ensure similar contact with the mucosal surfaces among subjects. Following the self-inoculation of virus, the virus contaminating the fingers of the left hand was eluted into 2 mL of virus-collecting broth for quantitative culture. Viral infection was assessed in the volunteers by culture of nasal lavage specimens collected on each of the 5 days following virus challenge. Blood samples were taken at 3 to 4 weeks after inoculation to determine seroconversion.

(v) Conduct of clinical trial 2. The residual virucidal activity of a skin cleanser wipe containing 4% pyrogallic acid formulated with 0.1% benzalkonium chloride was tested in clinical trial 2 and was also conducted by using a double-blind, randomized design. The negative control treatment was 62% ethanol. Benzalkonium chloride had been previously tested and was found to have no virucidal activity. Volunteers were randomly assigned to use the control preparation, the active preparation, or a untreated preparation. Thirty volunteers were treated with the control preparation, and 92 volunteers were treated with 4% pyrogallic acid. The study material was applied to hands with a towelette. Fifteen minutes later, when the fingers were completely dry, the fingertips of each hand of the 30 control subjects and 30 of the volunteers in the active treatment group were contaminated with 106 TCID<sub>50</sub> of rhinovirus type 39 in a volume of 100 μL. An additional 30 volunteers in the active group were challenged with virus 1 h after application, and the final group of 32 volunteers was challenged 3 h after application. These volunteers remained at the study site and were not allowed to use or wash their hands in the interval between the hand treatment and the virus challenge. The self-inoculation, recovery of virus from the hands, and assessment of infection were done as described above for the first clinical trial.

RESULTS

Residual antiviral efficacy on collagen substrates. A number of different organic acids were tested for residual antirhinoviral activity. Salicylic, pyrogallic, fumaric, and benzoic acids produced at least a 2-log reduction in viral titers (Table 1). These acids also had immediate antiviral activity, demonstrated by the inactivation of rhinovirus that was applied to the collagen substrate first, followed by the application of the acid solutions (data not shown). The immediate and residual antiviral activity demonstrated by salicylic, pyrogallic, fumaric, and benzoic acids was comparable to or exceeded that of the positive control (2% iodine). Chlorhexidine (4%), 1% tri-
TABLE 2. Effect of use (finger) versus nonuse (arm) sites and concentration of acid on antirhinoviral activity of salicylic and pyroglutamic acid

<table>
<thead>
<tr>
<th>Site and treatment</th>
<th>Virus titers at time interval (h) after treatment</th>
<th>0.2</th>
<th>1.0</th>
<th>3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Finger site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% iodine</td>
<td>&gt;2.7</td>
<td>1.7</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>2% salicylic acid</td>
<td>&gt;1.4</td>
<td>1.2</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>3.5% salicylic acid</td>
<td>&gt;1.8</td>
<td>1.5</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>4.0% pyroglutamic acid</td>
<td>&gt;2.0</td>
<td>1.2</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>4.68% pyroglutamic acid</td>
<td>&gt;2.2</td>
<td>1.1</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>1% salicylic + 3.5% pyroglutamic acid</td>
<td>&gt;2.5</td>
<td>2.0</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Arm site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% iodine</td>
<td>&gt;2.5</td>
<td>2.1</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>2% salicylic acid</td>
<td>&gt;2.1</td>
<td>1.9</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>

* Log reduction versus no treatment control.
* \( P > 0.05 \) compared to control by Fisher's least significant difference two-tailed test; all other titers were significantly reduced compared to the control.

Infection developed in 10 (32%) of the 31 control volunteers compared to 2 (7%) of the 27 volunteers in each of the active treatment groups. This reduction was statistically significant \( (P = 0.025 \) by two-sided Fisher's exact test) for both active preparations compared to the negative control. No adverse events related to the study material were reported in this experiment.

(ii) Clinical trial 2. In the second study, 30 volunteers were randomly assigned to use the control preparation, and 92 volunteers were assigned to use 4% pyroglutamic acid. This hand treatment had significant virucidal activity that persisted for at least 3 h after application. Viral control was detected on the hands of 30 out of 30 control volunteers tested 15 min after application of 62% ethanol. In contrast, viral control was detected on the hands of 5 out of 30 (17%), 8 out of 30 (27%), and 19 out of 32 (59%) volunteers 15 min, 1 h, and 3 h, respectively, after use of 4% pyroglutamic acid. These isolation rates were all significantly reduced \( (P < 0.0001 \) by two-sided Fisher's exact test) compared to the rate of isolation from volunteers in the negative control group. Quantitative cultures revealed that the control volunteers had \( 10^{1.4} \) TCID\(_{50} \) of rhinovirus recovered per milliliter of eluant fluid. Those volunteers who had detectable virus had \( 10^{1.0}, 10^{1.0}, \) and \( 10^{1.2} \) TCID\(_{50} \) of virus recovered per milliliter after 15 min, 1 h, and 3 h, respectively. The incidence of rhinovirus infection in the volunteers who used the control hand treatment was 9 out of 30 (30%). In those who used the active preparation, the incidence of infection was 4 out of 30 (13%) at both the 15-min and 1-h time points and 7 out of 32 (22%) at the 3-h time point. None of these reductions in isolation rate achieved statistical significance. Adverse events believed to be possibly or probably related to the study were reported by 10% of the volunteers who used the active treatment compared to 7% of placebo recipients. The most commonly reported adverse event was light irritation, reported by 5% of the active treatment group and 3% of the placebo group.

The length of time between application of the hand treatment and the hand contact with the conjunctival mucosa was not correlated with the incidence of eye irritation.

The efficacies of organic acid hand treatments appear to correlate with the amount of acid applied to the hands. The different amounts of acid applied by the preparations used in these two clinical trials permits assessment of the correlation between the amount of acid applied to the hands and the effect (at the 15-min time point) on recovery of virus from the hands and the prevention of rhinovirus infection (Table 3). These results indicate that both the elimination of virus from hands \( (r = -0.97, P = 0.02 \) by Spearman rank correlation) and the reduction in infection rate \( (r = -0.95, P = 0.02) \) are strongly correlated with the amount of acid applied to the hands.

TABLE 3. Correlation between amount of acid applied to the hands and removal of virus from hand and prevention of rhinovirus infection in human volunteers

<table>
<thead>
<tr>
<th>Acid used</th>
<th>No. of subjects</th>
<th>Amt of acid applied (mmol)</th>
<th>% Recovery of virus from hand</th>
<th>% Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>62% EtOH vehicle (trial 1 control)</td>
<td>31</td>
<td></td>
<td>90</td>
<td>32</td>
</tr>
<tr>
<td>62% EtOH vehicle (trial 2 control)</td>
<td>30</td>
<td></td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>4% pyroglutamic acid</td>
<td>30</td>
<td>.31</td>
<td>17</td>
<td>13</td>
</tr>
<tr>
<td>3.5% salicylic acid</td>
<td>27</td>
<td>.35</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>1% salicylic + 3.5% pyroglutamic acid</td>
<td>27</td>
<td>.48</td>
<td>0</td>
<td>7</td>
</tr>
</tbody>
</table>
DISCUSSION

The rhinoviruses are the most frequent cause of the common cold. Although these illnesses are generally self-limited and benign, they are associated with medically important complications. Otitis media generally occurs in the setting of a preceding viral upper respiratory infection, and as many as 30% of colds in children have been reported to be associated with this complication (19). In school-aged asthmatic children, 60 to 70% of asthma exacerbations are associated with a rhinovirus infection (14, 16). In spite of these important consequences of rhinovirus infection, little progress has been made on the development of effective treatments for these infections.

In spite of the frequency of rhinovirus infections, person-to-person transmission of rhinovirus is relatively inefficient. A small study of married couples found transmission from one housemate to the other in only about one-third of the cases (1). Similarly, studies in an experimental model suggested that approximately 200 h of contact with infected and symptomatic “donors” was required for transmission of infection to 50% of susceptible “recipients” (15). Rhinovirus appears to spread by both direct contact and large-particle aerosol (6), although the relative contribution of these different mechanisms is not clear. The role of direct contact with hand contamination in the spread of colds was examined in a study in which iodine was used as a virucidal agent (11). In this study, the incidence of common colds was reduced by 40% in mothers who were treating their hands with iodine compared to mothers who used placebo. This study suggested that an effective virucidal agent that had residual activity on the hands for 2 to 3 h could have an important impact on the spread of colds in the natural setting.

Our studies suggest that organic acids commonly used in over-the-counter skin care and cosmetic products have substantial virucidal activity against rhinoviruses that persists for 2 to 3 h after application. Persistent acidification of the skin surface appears to be the mechanism for the virucidal activity of the organic acids reported here. The amount of acid applied to the hands correlated directly with the prevention of infection in the deliberate infection model.

The utility of our observation in the natural setting remains to be determined. We have demonstrated that hand treatment reduces hand contamination with rhinovirus and can prevent colds under the controlled conditions of the experimental model. The results in the experimental model, however, cannot be extrapolated to the natural setting. In this study, the volunteers were not allowed to use their hands in the interval between the hand treatment and the virus challenge, so the effect of normal use of the hands on the virucidal activity of these organic acids is not known. Similarly, the virus challenge method used in these experiments may not simulate the natural setting in all aspects. The amount of virus recovered from the hands of rhinovirus-infected individuals is generally less than 20 TCID50 per 2 ml (17), comparable to the amount of virus recovered from the hands of the volunteers in our model. However, the effect of nasal secretions that would be transferred with the virus in the natural setting on the activity of the acids or on the transmission of virus was not tested in our model.

The compounds used in this study are commonly used in over-the-counter beauty products and are generally considered safe. Whether alteration of the concentration of these compounds to enhance the virucidal activity would be associated with safety concerns is not known. There were no important adverse events in the small numbers of subjects with limited exposure to the treatments in our study.

We believe that the observation that commonly used organic acids have virucidal activity for rhinoviruses that persists for hours after application has potentially important implications for the prevention of these infections. In light of the ubiquitous occurrence and medically important consequences of these infections and the lack of effective alternative treatments, this simple and apparently safe intervention deserves further study in the natural setting.

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REFERENCES